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Cyclin D-Cdk4 Is Not a Master Regulator of Cell Multiplication in *Drosophila* Embryos

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Summary

Inactivation of Cyclin E-Cdk2 is essential for a timely arrest of the epidermal cell proliferation program during *Drosophila* embryogenesis [1, 2]. E-type cyclin-cdk complexes are thought to be activated by D-types titrating away inhibitors and inducing cyclin E transcription by activating E2F transcription factors via Rb phosphorylation [3]. Therefore, we have analyzed whether the developmentally controlled inactivation of Cyclin E-Cdk2 required for the epidermal cell proliferation arrest occurs as a consequence of Cyclin D-Cdk4 inactivation. However, preventing Cyclin D-Cdk4 inactivation by overexpression has a minimal effect on *Cyclin E* expression and does not interfere with the initial G1 arrest, while it readily induces the E2F target *RnrS* in arresting epidermal cells. Prolonged *Cyclin D-Cdk4* overexpression eventually interferes with maintenance of quiescence in some cells. Moreover, in *Cdk4* mutant embryos, some *RnrS* expression is still induced by *Cyclin E* overexpression, and endogenous *Cyclin E* expression as well as cell cycle progression is not affected, except for late aspects of the endoreduplication program. These findings argue against the proposed necessity of complete Rb inactivation by sequential phosphorylation by D- and E-type cyclin-cdk complexes. They demonstrate that Cyclin D-Cdk4 does not function as the master regulator of the embryonic cell proliferation program.

Results and Discussion

Based on the results obtained in vitro and with cultured mammalian cells, cyclin D-cdk4/6 complexes are expected to play a major role in the regulation of cell proliferation during development. In contrast to mammals, only single genes for Cyclin D and its partner kinase Cdk4 are present in *Caenorhabditis elegans* and *Drosophila melanogaster*, which also have Rb and E2F homologs. Genetic analyses in these organisms have clearly demonstrated that the D-type cyclin complexes do not provide an obligatory function that is required in each and every cell cycle for entry into S phase. In *Caenorhabditis*, *cyclin D* and *cdk4* appear to be dispensable for embryonic development [4, 5], and D-type complexes are essentially absent in undifferentiated mouse ES cells and early embryos [6]. In *Drosophila*, a few escapers develop to the adult stage even in the complete absence of parental and zygotic *Cdk4*$^{+}$ function [7], indicating that alternative mechanisms controlling progression through G1 do exist. While not essential, *Drosophila* Cyclin D-Cdk4 might nevertheless contribute to the control of the embryonic cell proliferation program, and it might have to be inhibited to allow exit from the cell cycle in wild-type embryos. Therefore, we have studied the role of Cyclin D-Cdk4 during embryogenesis in further detail.

In mammals, the transcription of D-type cyclin genes has been shown to be regulated by extracellular signals [8, 9]. Therefore, the distribution of *Cyclin D* transcripts during *Drosophila* embryogenesis might be expected to be highly dynamic. However, in situ hybridization experiments (see the Supplementary Material available with this article online) revealed a fairly uniform distribution of *Cyclin D* transcripts throughout the embryo, although signals were somewhat higher in the mesoderm during germband-extended stages and in the midgut after germband retraction. *Cdk4* probes gave very similar results. The pattern of *Cyclin D* and *Cdk4* transcription, therefore, does not anticipate the pattern of S phase comparable to *Cyclin E*. Distinct pulses of *Cyclin E* [1, 10], but not *Cyclin D*, and *Cdk4* expression anticipate, in particular, the S phases during the endoreduplication cycles, which occur in a reproducible tissue-specific pattern during late embryogenesis [11]. By immunofluorescence, our antibodies were unable to detect the low levels of expression from the endogenous genes, while they readily generated specific signals after *Cyclin D* and *Cdk4* overexpression (see the Supplementary Material). However, immunoblotting experiments with total embryo extracts prepared at different times after egg deposition confirmed the presence of Cyclin D and Cdk4 protein during embryogenesis (see the Supplementary Material).

Genetic interactions have indicated that Cyclin D-Cdk4 inhibits the function of the *Drosophila* Rb homolog *RBF* [7, 12]. In addition, RBF has been shown to inhibit the expression of E2F target genes like *RnrS* [13–15], which encodes a subunit of ribonucleotide reductase. Cyclin D-Cdk4 activity, therefore, is expected to induce *RnrS* transcription. By in situ hybridization, we evaluated whether *UAS-Cyclin D* and *UAS-Cdk4* expression using *prd-GAL4*, which directs *UAS* transgene expression in alternating segments [16], results in increased *RnrS* transcript levels. Our *RnrS* probe clearly detected increased signals in the *Cyclin D-Cdk4*-overexpressing segments, while the intervening segments displayed the normal pattern of *RnrS* expression (Figures 1A and 1C). Importantly, signal intensities in overexpressing regions were comparable to those within focal planes of the nervous system, reflecting normal endogenous *RnrS* expression (data not shown). Overexpression of *Cyclin D* and *Cdk4*, therefore, is sufficient to trigger physiological levels of *RnrS* transcription. Induction of *RnrS* transcription was not observed when *UAS-Cyclin D* and *UAS-Cdk4* were expressed individually.

Apart from *RnrS*, *Cyclin E* has also been shown to be

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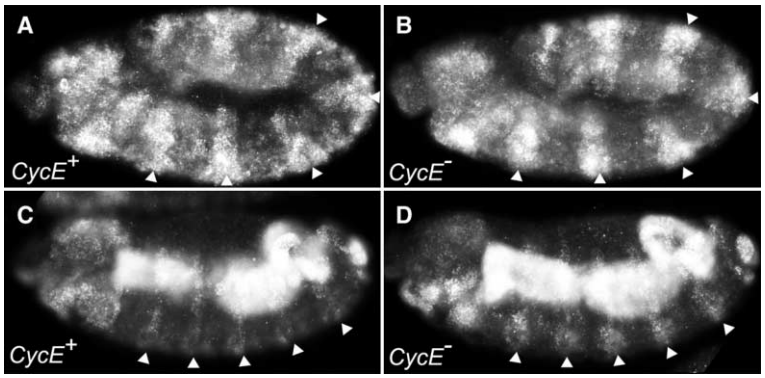

Figure 1. *Cyclin D-Cdk4* Overexpression Induces Expression of the E2F Target Gene *RnrS* Independently of *Cyclin E*

(A–D) The distribution of *RnrS* transcripts was analyzed by in situ hybridization using fluorescent probe detection combined with anti-β-galactosidase labeling (not shown), allowing for the identification of (A and C) *Cyclin E*$^+$ and (B and D) *Cyclin E*$^-$ sibling embryos carrying *prd-GAL4*, *UAS-Cyclin D*, and *UAS-Cdk4* at (A and B) stage 11 and (C and D) stage 13. Arrowheads indicate some of the regions with ectopic *RnrS* transcripts induced by *Cyclin D-Cdk4* overexpression.

an E2F target in *Drosophila* [17, 18] and in mammalian cells [19–21]. Similarly, cyclin A has been proposed to be induced by E2F in mammalian cells [3, 22, 23]. However, in situ hybridization experiments with *Cyclin E* and *Cyclin A* probes did not reveal a distinct increase in signal intensity within *Cyclin D-Cdk4*-overexpressing regions. Occasionally, very weak signals were detectable in these regions just above background (data not shown). Nevertheless, it is clear that *Cyclin D-Cdk4* overexpression cannot induce normal levels of *Cyclin E* or *Cyclin A* expression, in contrast to the results with *RnrS*.

Rb phosphorylation by cyclin D-cdk4 in mammalian cells has been proposed to allow the recruitment of cyclin E-cdk2, which can then phosphorylate a critical serine residue of Rb, provoking the dissociation of Rb from E2F and thereby transcriptional activation by free E2F [24]. Moreover, complete activation of E2F target genes has previously been suggested to depend on sequential activity of cyclin D-cdk4 and cyclin E-cdk2 [24–30]. Therefore, we analyzed whether induction of *RnrS* transcription by *Cyclin D-Cdk4* overexpression in *Drosophila* embryos is dependent on *Cyclin E*. For these experiments, we crossed *UAS-Cyclin D*, *UAS-Cdk4*, and *prd-GAL4* into *Cyclin E* mutant embryos. Embryos lacking zygotic expression of *Cyclin E* are known to progress normally through the first 16 cycles of embryogenesis with the help of a maternal *Cyclin E* contribution. However, this maternal contribution is no longer sufficient for progression through S phase 17 [1]. Thus, we analyzed whether *Cyclin D-Cdk4* overexpression at this and during later stages is still capable of increasing *RnrS* transcript levels in *Cyclin E* mutants. Ectopic *RnrS* transcripts were readily detected in *Cyclin E* mutants in regions expressing the *UAS* transgenes (Figures 1B and 1D). Our results indicate, therefore, that *RnrS* induction by Cyclin D-Cdk4 is not dependent on Cyclin E-Cdk2.

A few embryos can develop until the adult stage without maternal and zygotic *Cdk4*$^+$ function [7]. Therefore, *RnrS* transcription is unlikely to be absolutely dependent on Cyclin D-Cdk4, even though it is clearly induced by *Cyclin D-Cdk4* overexpression. In situ hybridization directly demonstrated that *RnrS* transcripts are present during embryogenesis also when maternal and zygotic *Cdk4*$^+$ function is lacking (Figures 2A–2E). However, transcript levels were clearly decreased in *Cdk4* mutants, as was most apparent within a central domain of the midgut (Figures 2D and 2E). In wild-type embryos, a prominent pulse of *RnrS* expression is observed preceding endoreduplication in this as well as in the other regions ([10], also see Figure 2C).

We also analyzed *Cyclin E* expression and BrdU incorporation reflecting progression through S phase in embryos lacking maternal and zygotic *Cdk4*$^+$ function. In situ hybridization with a *Cyclin E* probe as well as BrdU labeling did not reveal obvious differences between *Cdk4* mutants and control embryos until germband retraction. However, after germband retraction, *Cyclin E* transcript levels were decreased in the absence of zygotic *Cdk4*$^+$ function (and irrespective of the maternal *Cdk4* contribution) specifically in endoreduplicating tissues, while normal levels were present in the mitotically proliferating nervous system (Figures 2G, 2I, and 2J). Similarly, BrdU incorporation was found to be only affected in endoreduplicating tissues of late *Cdk4* mutant embryos. While BrdU incorporation appeared normal in the anterior and posterior midgut (Figure 2L), it occurred less efficiently and in fewer cells of the central midgut (Figures 2N and 2O). During wild-type embryogenesis, the central midgut endoreduplicates at a later developmental stage than the other gut regions [11]. At this later stage, we still observed abnormally high levels of BrdU incorporation in the other gut regions of *Cdk4* mutant embryos (Figures 2N and 2O). These findings indicate that progression through endocycle S phases in the gut is slow or even fails in the absence of zygotic *Cdk4*$^+$ function. However, complete loss of both maternal and zygotic *Cdk4*$^+$ function did not appear to affect *Cyclin E* transcript levels and S phase progression in the nervous system (Figures 2J and 2O). The results concerning *RnrS* and *Cyclin E* transcription as well as the pattern of S phases observed in *Cdk4* mutants, therefore, are similar to those described previously in *E2f1* and *DP* mutants [31, 32]

The surprisingly normal mitotic cell cycle progression in *Cdk4* mutants might reflect a partial functional overlap between Cyclin D-Cdk4 and Cyclin E-Cdk2. Genetic interactions support this notion, and overexpression of *Cyclin E* from a heat-inducible transgene can clearly induce *RnrS* transcription [7, 17, 33]. Using *UAS-Cyclin E* in combination with *prd-GAL4*, we also observed ectopic *RnrS* transcripts in *Cyclin E*-overexpressing regions (Figures 3C and 3D), although somewhat less prominently than after *Cyclin D-Cdk4* overexpression. Moreover, with *UAS-Cyclin E*, ectopic *RnrS* expression was only observed before germband retraction, in contrast to the results from experiments with *UAS-Cyclin*

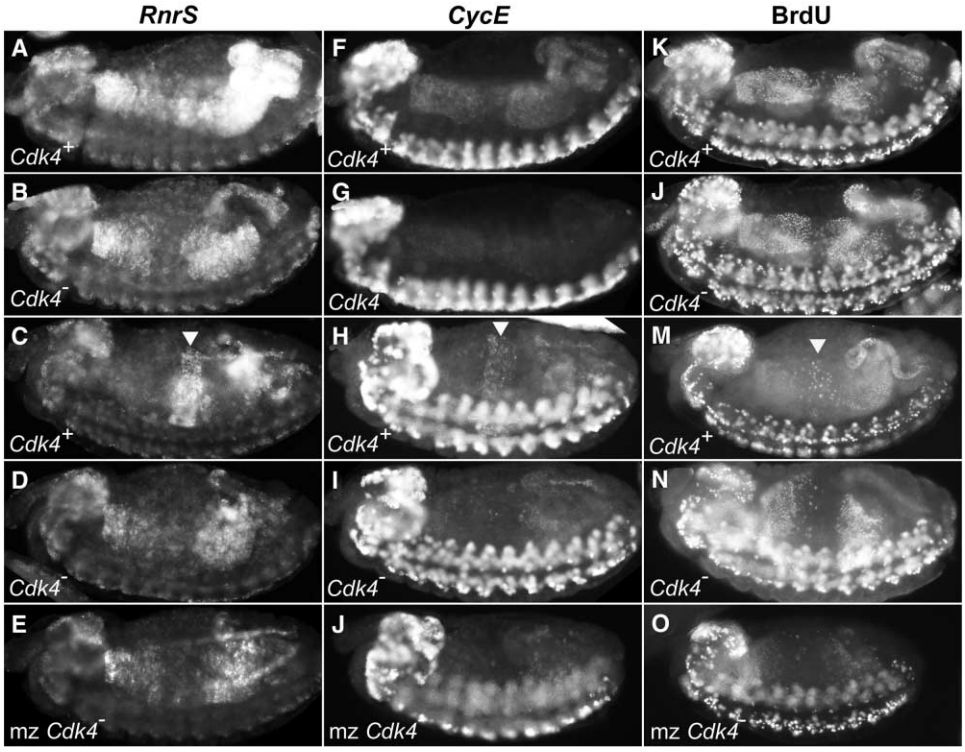

Figure 2. S Phase Gene Expression and S Phase Progression in *Cdk4* Mutants

(A–J) The distribution of (A–E) *RnrS* and (F–J) *Cyclin E* transcripts was analyzed by in situ hybridization using fluorescent probe detection. (K–O) In addition, the distribution of cells in S phase was analyzed by BrdU pulse labeling. Embryos were at (A, B, F, G, K, and L) stage 13 or (C–E, H–J, and M–O) stage 14 and contained (A, C, F, H, K, and M) $Cdk4^{+}$ ($Cdk4^{+}$) or lacked $Cdk4^{+}$ either (B, D, G, I, L, and N) zygotically ($Cdk4^{-}$) or both (E, J, and O) maternally and zygotically (mz $Cdk4^{-}$). Arrowheads indicate the central midgut domain.

D and *UAS-Cdk4*. We speculated that the failure of *Cyclin E* overexpression to induce *RnrS* expression in germband-retracted embryos might be caused by the expression of the specific Cyclin E-Cdk2 inhibitor p27Dacapo, which is known to start just before germband retraction [2, 34]. However, ectopic *RnrS* transcription was not prolonged in *dacapo* mutant embryos with *UAS-Cyclin E* and *prd-GAL4* (data not shown).

We further tested whether *RnrS* induction by *Cyclin E* overexpression is dependent on Cdk4. Therefore, we crossed *UAS-Cyclin E* and *prd-GAL4* into mutant embryos lacking both maternal and zygotic $Cdk4^{+}$ function.

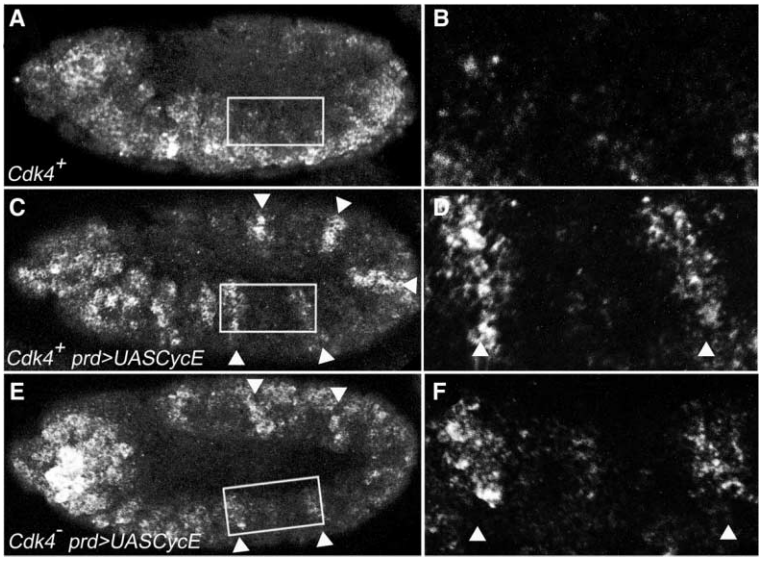

Figure 3. *Cyclin E* Overexpression Induces Expression of the E2F Target Gene *RnrS* in $Cdk4^{+}$ and with Reduced Efficiency in $Cdk4^{-}$ Embryos

The distribution of *RnrS* transcripts was analyzed by in situ hybridization using fluorescent probe detection in either (A and B) wild-type or (C–F) *prd-GAL4*, *UAS-Cyclin E* embryos with (C and D) $Cdk4^{+}$ or without (E and F) maternal and zygotic $Cdk4^{+}$ function. Arrowheads indicate some of the regions with ectopic *RnrS* transcripts induced by *Cyclin D-Cdk4* overexpression. The regions boxed in (A), (C), and (E) are shown in (B), (D), and (F).

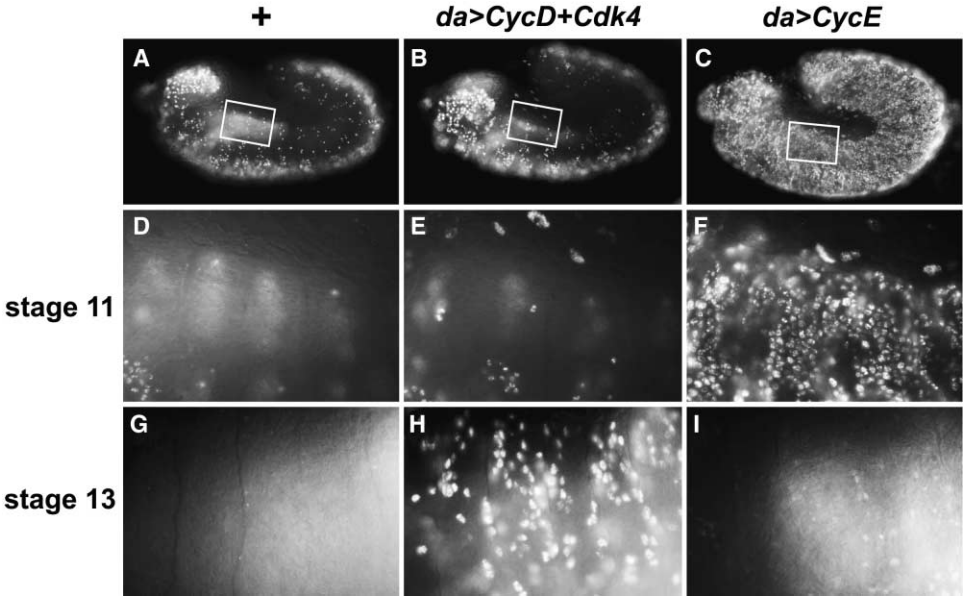

Figure 4. *Cyclin D-Cdk4* Overexpression Interferes with Maintenance but Not with Entry into Quiescence

(A–F) (A, D, and G) Control embryos and *da-GAL4* embryos with (B, E, and H) *UAS-Cyclin D* and *UAS-Cdk4* or (C, F, and I) *UAS-Cyclin E* were pulse labeled with BrdU at (A–F) stage 11 and (G–I) stage 13. (D–I) High magnification views illustrate anti-BrdU labeling in the epidermis. The regions boxed in (A)–(C) are shown in (D)–(F).

In these embryos, we observed a slight but distinct increase of signal intensity in the *Cyclin E*-overexpressing regions after in situ hybridization with the *RnrS* probe (Figures 3E and 3F). These signals were weaker than those detected in sibling embryos with a paternally inherited $Cdk4^{+}$ gene (Figures 3C and 3D). Our results indicate, therefore, that *Cyclin E* overexpression also induces *RnrS* transcription in the absence of Cdk4, although with reduced efficiency.

UAS-Cyclin D and *UAS-Cdk4* expression with *prd-GAL4* induces *RnrS* transcription during the developmental stages when the epidermal cells normally exit from the mitotic cell cycle and become mitotically quiescent. Analogous expression of *UAS-Cyclin E*, which can also raise *RnrS* transcript levels, has been shown to prevent epidermal cells from leaving the mitotic cell cycle at the appropriate developmental stage. Epidermal cells overexpressing *Cyclin E* progress through an additional division instead of arresting in G1 after mitosis 16, which is the terminal division during wild-type embryogenesis [1] (Figures 4C and 4F). Therefore, we analyzed whether *Cyclin D-Cdk4* overexpression also prevents a timely epidermal cell proliferation arrest. In addition to *prd-GAL4* (data not shown), we also used *da-GAL4* for *UAS-Cyclin D* and *UAS-Cdk4* expression, because *da-GAL4* is expressed continuously (and ubiquitously), in contrast to *prd-GAL4*, which is no longer expressed in germband-retracted embryos. BrdU pulse labeling immediately after mitosis 16 indicated that *Cyclin D-Cdk4* overexpression does not prevent entry into G1 (Figures 4B and 4E). Interestingly, BrdU pulse labeling about 3 hr later revealed some labeled cells within the *Cyclin D-Cdk4*-overexpressing regions (Figure 4H), indicating that maintenance of quiescence is compromised.

The same findings have been made in embryos lacking both maternal and zygotic *RBF* function [14]. In these *RBF* mutant embryos, *Cyclin E* has been shown to become reexpressed in some of the epidermal cells, suggesting an explanation for the observed exit from the G1 arrest. As indicated above, *Cyclin D-Cdk4* overexpression can also induce *Cyclin E* expression, although only to barely detectable levels. To evaluate whether *Cyclin E* is required for cell cycle reentry in *Cyclin D-Cdk4*-overexpressing epidermal cells, we repeated the experiments in *Cyclin E* mutants (data not shown). In these mutants, *Cyclin D-Cdk4* overexpression did not result in ectopic BrdU incorporation, indicating that Cyclin E is required for reentry into S phase. These findings emphasize the fact that the maintenance of quiescence in postmitotic epidermal cells is highly dependent on very tight inhibition of Cyclin E, whose expression can be induced via the Cyclin D-Cdk4-RBF-E2F pathway, although presumably only with minor efficiency.

In conclusion, while *Cyclin D-Cdk4* overexpression interferes in some cells with maintenance of quiescence, it does not preclude an initial timely entry into proliferative quiescence. Moreover, the effects of *Cyclin D-Cdk4* and *Cyclin E* overexpression in *Cyclin E* and *Cdk4* mutant embryos, respectively, cannot be easily reconciled with an obligatory sequential action of D- and E-type cyclin-cdk complexes, which has been postulated for some classes of E2F target genes based on evidence obtained in vitro and in mammalian cell culture systems [3, 24] (see the Supplementary Material for a more extensive discussion). Cyclin D-Cdk4 plays a minor role in the control of Cyclin E-Cdk2 activity and cell cycle progression during the embryonic cell division cycles. These cycles are characterized by a relaxed coupling

to cell growth because of the abundant maternal stores that are stockpiled in the egg. However, Cyclin D-Cdk4 is partially required for progression through endoreduplication cycles late in embryogenesis, which might already be triggered by nutrients extracted from the yolk by the newly formed gut, similar to the larval endoreduplication cycles, which are clearly nutrient dependent [35]. Moreover, consistent with our proposal that Cyclin D-Cdk4 complexes are primarily involved in the regulation of growth rates [7, 12], we have previously described that oogenesis and imaginal development, which are tightly coupled to growth, are most severely affected in *Cdk4* mutants.

Experimental Procedures

Experimental details are described in the Supplementary Material. We point out that the progeny of $Cdk4^{3}$ mutant females are severely affected by maternal effect phenotypes (supplementary material of [7]). Only a few percent of the progeny progress normally through the syncytial division cycles, while the other embryos are affected by division errors to a very variable extent. Because of these early maternal effect defects, most embryos display highly variable morphological abnormalities during the later embryonic stages analyzed here. In the figures shown here, we present embryos with essentially normal morphology. The observations in the more abnormal embryos also support our conclusions.

For the classification of embryo genotypes, we used balancer chromosomes with transgenes driving *lacZ* expression. In situ hybridization combined with anti-β-galactosidase double labeling was performed using tyramide signal amplification (TSA Kit, NEN) for probe detection by fluorescence [36]. Our comparison of $CycE^{+}$ and $CycE^{-}$, as well as $Cdk4^{+}$ and $Cdk4^{-}$ embryos, therefore, relied on embryos that were fixed and processed together and identified by anti-β-galactosidase double labeling.

Supplementary Material

Our results from experiments addressing the expression of *Cyclin D* and *Cdk4* during embryogenesis by immunoblotting and in situ hybridization are described in two Supplementary Figures (Figures S1 and S2). Moreover, our findings are more extensively discussed in relation to previous work in *Drosophila* and mammalian organisms. Finally, the Experimental Procedures and the additional figures are available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

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